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Identification of glyphosate-resistant Italian ryegrass (*Lolium multiflorum*) in Oregon

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A suspected glyphosate-resistant Italian ryegrass biotype was collected from a filbert orchard near Portland, OR, where glyphosate was applied multiple times per year for about 15 yr. Greenhouse studies were conducted to determine if this biotype was glyphosate resistant. The plants were sprayed with glyphosate (0.01 to 3.37 kg ae ha^{-1}) 14 d after planting and shoot biomass was determined 3 wk after herbicide treatment. Based on the dose–response experiments conducted in the greenhouse, the suspected Italian ryegrass biotype was approximately fivefold more resistant to glyphosate than the susceptible biotype. Plants from both susceptible and resistant biotypes were treated with glyphosate (0.42 and 0.84 kg ha^{-1}) and shikimic acid was extracted 12, 24, 48, and 96 h after treatment. The susceptible biotype accumulated between three and five times more shikimic acid than did the resistant biotype. Leaf segments from both susceptible and resistant biotypes were incubated with different glyphosate concentrations (0.5 to 3000 μM) for 14 h under continuous light. Shikimic acid was extracted from each leaf segment and quantified. At a concentration up to 100 μM, leaf segments from the susceptible biotype accumulated more shikimic acid than leaf segments from the resistant biotype. The *epsps* gene was amplified and sequenced in both susceptible and resistant biotypes; however, no amino acid change was found in the resistant biotype. The level of resistance in this biotype is similar to that reported for a glyphosate-resistant Italian ryegrass biotype from Chile.

Nomenclature: Glyphosate; Italian ryegrass, *Lolium multiflorum* Lam. LOLMU; filbert, *Corylus avellana* L.

Key words: Glyphosate resistance, herbicide resistance, resistance mechanism.

Glyphosate is a broad-spectrum, nonselective herbicide that has been widely used for vegetation control in plantation crops, no-tillage systems, and nonagricultural situations since its commercialization in the 1970s (Baylis 2000). Glyphosate is also currently used in glyphosate-resistant crops, such as soybean [*Glycine max* (L.) Merr.], cotton (*Gossypium hirsutum* L.), canola (*Brassica napus* L.), and corn (*Zea mays* L.) for selective weed control (Shaner 2000).

Glyphosate inhibits the enzyme 5-enolpyruvylshikimate-3-phosphate (EPSP) synthase (EC 2.5.1.19). EPSP synthase catalyzes the conversion of shikimate-3-phosphate and phosphoenolpyruvate (PEP) to yield EPSP and inorganic phosphate in the shikimate pathway (Geiger and Fuchs 2002). Glyphosate is a competitive inhibitor of PEP; it occupies the binding site of PEP, mimicking an intermediate state of the ternary enzyme–substrates complex (Schönbrunn et al. 2001). The inhibition of EPSP synthase results in shikimate accumulation and prevents the biosynthesis of the aromatic amino acids, phenylalanine, tyrosine, and tryptophan.

Glyphosate was introduced in 1974 (Woodburn 2000). Despite its widespread and long-term use, evolved resistance to glyphosate was not reported until 1996 (Pratley et al. 1996). The unique properties of glyphosate, such as its mode of action, chemical structure, limited metabolism, and lack of residual activity in soil, may explain why it took so long for glyphosate resistance to evolve in weed populations (Bradshaw et al. 1997). Other factors that may be just as important include the high cost of glyphosate until recently and that it was often used in combination with other herbicides.

Today, evolved resistance to glyphosate has been reported in six weed species worldwide. The first case was detected in rigid ryegrass (*Lolium rigidum* L.) in Australia (Powles et al. 1998; Pratley et al. 1999), followed by goosegrass [*Eleusine indica* (L.) Gaertn.] in Malaysia (Tran et al. 1999; Lee and Ngim 2000), horseweed [*Conyza canadensis* (L.) Cronq.] in the United States (Koger et al. 2004; Main et al. 2004; VanGessel 2001), and Italian ryegrass in Chile (Perez and Kogan 2003). Glyphosate resistance also has been detected in rigid ryegrass in California (Simarmata et al. 2003) and South Africa, in hairy fleabane [*Conyza bonariensis* (L.) Cronq.] in South Africa and Spain (Urbano et al. 2005), in common ragweed (*Ambrosia artemisiifolia* L.) in Missouri (Sellers et al. 2005), and in buckhorn plantain (*Plantago lanceolata* L.) in South Africa (Heap 2005).

In 2003, glyphosate at 1.68 kg ae ha^{-1} failed to effectively control an Italian ryegrass population from a filbert orchard near Portland, OR (Perez-Jones et al. 2004). Glyphosate had successfully controlled weeds in this orchard during the previous 15 yr. Greenhouse and laboratory experiments were conducted to determine if the Italian ryegrass biotype was resistant to glyphosate. Additional objectives of this research were to characterize the level of resistance to glyphosate in the Italian ryegrass biotype on a whole-plant basis, to investigate shikimic acid accumulation in response to glyphosate application, and to compare the amino acid sequences of the *epsps* gene between the susceptible and the resistant Italian ryegrass biotypes.

Materials and Methods

Plant Material

Italian ryegrass was collected from a filbert orchard located near Portland, OR, in 2003. This site had been intensively treated with glyphosate, two to three applications per year at 1.68 kg ha^{-1}, during the last 15 yr. The collected plants that survived a field application of glyphosate at 1.68 kg ha^{-1} were grown in the greenhouse and seeds were produced. A known susceptible Italian ryegrass biotype collected in the Willamette Valley, OR, was included as a control.

Whole-Plant Bioassay

Seeds of both susceptible and resistant Italian ryegrass biotypes were planted in 267-ml plastic pots containing commercial potting mix[1]. Plants were grown in the greenhouse under 25/20 C day/night temperature and a 16-h photoperiod. Plants at the three-leaf stage were sprayed with glyphosate[2] (0.01, 0.05, 0.11, 0.21, 0.42, 0.84, 1.68, and 3.37 kg ha^{-1}) using an 8003 even flat fan nozzle and overhead compressed-air sprayer calibrated to deliver 187 L ha^{-1}. Shoot biomass was harvested 3 wk after herbicide treatment, dried at 70 C for 48 h, and weighed. Biomass data are reported as percent of the untreated control.

Whole-Plant Shikimic Acid Bioassay

Shikimic acid extraction was performed according to Singh and Shaner (1998) with some modifications. Plants from both susceptible and resistant biotypes were grown in the greenhouse and treated at the three-leaf stage with glyphosate[2] (0.42 and 0.84 kg ha^{-1}) as described previously. Plant leaves (second and third leaf) were harvested for shikimic acid extraction 12, 24, 48, and 96 h after treatment. Leaf tissues were chopped and 0.050-g fresh wt samples were placed in 1.5-ml tubes containing 1 ml 0.25 N HCl. The samples were immediately mixed, placed at −20 C until frozen, thawed at room temperature, and incubated at 37 C for 45 min. Shikimic acid was measured spectrophotometrically using the method of Cromartie and Polge (2000). Three 25-μl aliquots per sample were mixed with 100 μl 0.25% periodic acid/0.25% sodium(meta)periodate solution in different wells in a 96-well plate. The plate was incubated at 37 C for 30 min to allow shikimic acid oxidation. After incubation, the samples were mixed with 100 μl 0.6 N NaOH/0.22 M Na_2SO_3 and optical density was measured spectrophotometrically at 380 nm. Shikimic acid in micrograms per gram fresh weight was determined based on a standard curve. The standard curve was determined using untreated plants and known concentrations of shikimic acid.

Leaf-Segment Shikimic Acid Bioassay

The effect of glyphosate on shikimic acid accumulation in Italian ryegrass was also determined using leaf segments following the method of Shaner et al. (2005). Leaf segments (0.5 cm) were removed from young leaves (second and third leaf) of susceptible and resistant biotypes and were placed in 96-well plates containing 100 μl 10 mM $NH_4H_2PO_4$ (pH 4.4) per well at different glyphosate[2] concentrations (0.5, 1, 3, 5, 10, 30, 50, 100, 300, 500, 1,000, and 3,000 μM). The leaf segments were incubated for 14 h under continuous light (100μM m^{-2} s^{-1}) at 25 C. The samples were placed at −20 C until frozen and then thawed at room temperature to disrupt the leaf tissue. Then each well received 25μl 0.25 N HCl and plates were incubated at 60 C for 30 min or until the tissue was digested. Aliquots of 25 μl were mixed with 100 μl of 0.25% periodic acid/0.25% sodium(meta)periodate solution in a different plate, and shikimic acid concentration was determined as described previously.

EPSP Synthase Gene Sequencing

Total RNA was extracted from leaf tissue of both susceptible and resistant Italian ryegrass biotypes using an RNA isolation kit.[3] First strand complementary DNA (cDNA) synthesis was performed from total RNA using a first strand synthesis system[4] and the oligo$(dT)_{20}$ primer. Degenerate primers were designed based on homologous regions of EPSP synthase gene sequences of goosegrass, rigid ryegrass, and rice (*Oryza sativa* L.) (GeneBank Accession numbers AY157642, AF349754, and AF413081, respectively). Polymerase chain reaction (PCR) was conducted to amplify the *epsps* gene in a 50-μl reaction using a Primus96 plus thermocycler.[5] The reaction mixture contained 1× PCR buffer, 0.2 μM of each primer, 0.2 mM of each deoxynucleotide, 1 unit of *Taq* DNA polymerase,[6] and 80 to 100 ng of template DNA. The cycling program consisted of one denaturation step of 3 min at 94 C, 35 cycles of 30 sec at 94 C, 30 sec at 50 C, and 1 min 30 sec at 72 C, followed by a final extension step of 10 min at 72 C. One pair of primers amplified a 1.2-kb fragment (sense: 5′-TSCAGCCCATCA RGGAGATCT-3′; antisense: 5′-TGCCATGGCCATGCG GTGRTC-3′) of the *epsps* gene from both biotypes. The amplified cDNA fragments were cloned using a cloning kit,[7] purified using a PCR purification kit,[8] and sequenced using an automatic DNA sequencer[9] with fluorescent dye–labeled dideoxynucleotides. Multiple clones per biotype were sequenced to exclude PCR errors and aligned.

Statistical Analysis

Dose–response curves for the whole-plant bioassay were obtained by a nonlinear regression using the log-logistic equation (Seefeldt et al. 1995; Streibig 1988; Streibig et al. 1993):

$$y = C + \frac{D - C}{1 + \left(\frac{x}{GR_{50}}\right)^{b}} \quad [1]$$

where y represents shoot dry weight (percentage of control) at herbicide rate x, C is the mean response at very high herbicide rate (lower limit), D is the mean response when the herbicide rate is zero (upper limit), b is the slope of the line at GR_{50}, and GR_{50} is the herbicide rate required for 50% growth reduction. The regression parameters from the susceptible and the resistant Italian ryegrass biotypes were obtained using Sigma Plot®[10] and compared to test significant differences with a sum of squares reduction test. The level of resistance was determined by calculating the ratio of the GR_{50} of the resistant biotype to the GR_{50} of the susceptible biotype.

I_{50} values were determined by calculating the herbicide

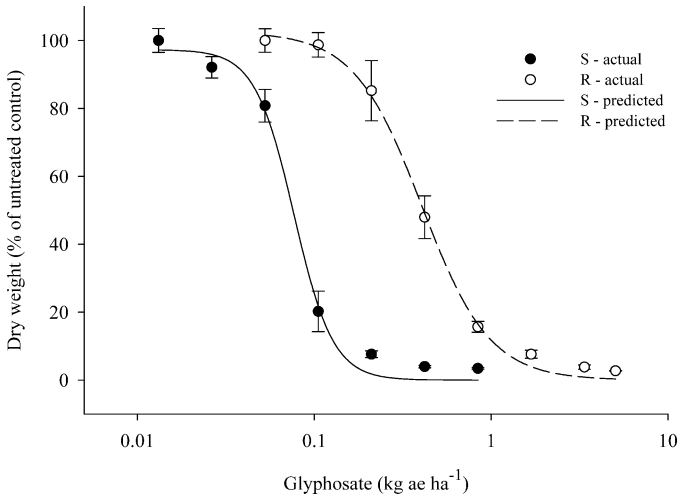

FIGURE 1. Shoot biomass of glyphosate-susceptible (S) and glyphosate-resistant (R) Italian ryegrass biotypes as affected by glyphosate rate. Symbols and lines represent actual and predicted growth responses, respectively. Vertical bars represent ± standard errors of the mean.

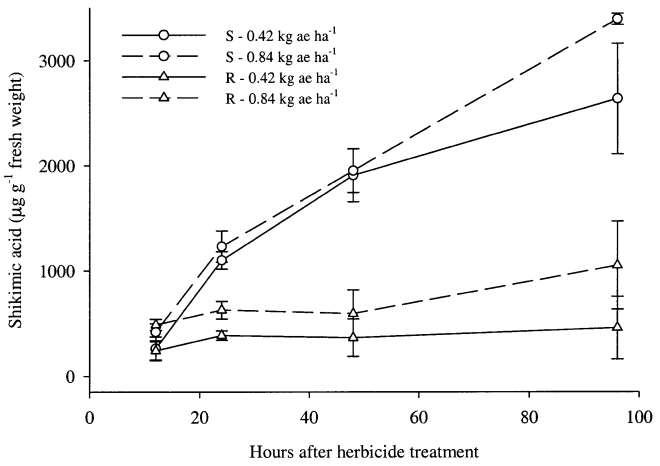

FIGURE 2. Shikimic acid accumulation in shoots of glyphosate-susceptible (S) and glyphosate-resistant (R) Italian ryegrass biotypes following the application of glyphosate (0.42 and 0.84 kg ae ha^{-1}). Vertical bars represent ± standard errors of the mean.

concentration required to inhibit enzyme activity by 50%, based on the leaf-segment shikimic acid bioassay. Analysis of variance for the whole-plant bioassay and shikimic acid bioassay studies showed no significant interaction between experiments and treatments; therefore, data from repeated experiments were combined. Data are presented as means of two experiments with four replications each.

Results and Discussion

Whole-Plant Bioassay

A differential response to glyphosate between the two Italian ryegrass biotypes was observed (Figure 1). The biotype collected from the filbert orchard will therefore be referred to as the resistant biotype. Shoot dry weight relative to the untreated control decreased with increasing glyphosate rate in both biotypes. However, at 0.1 kg ha^{-1}, shoot growth was reduced in the susceptible biotype to 20% of the untreated control, whereas in the resistant biotype it was reduced to only 98%. The GR_{50} for the resistant biotype (GR_{50} = 0.41 ± 0.02 kg ha^{-1}) to glyphosate was approximately fivefold greater than for the susceptible biotype (GR_{50} = 0.08 ± 0.01 kg ha^{-1}). This level of glyphosate resistance is similar to that observed in Italian ryegrass in Chile (Perez and Kogan 2003) and slightly lower than the level of resistance observed in rigid ryegrass in Australia, which was 7- to 11-fold (Powles et al. 1998; Pratley et al. 1999).

Whole-Plant Shikimic Acid Bioassay

At 0.42 and 0.84 kg ha^{-1}, the susceptible biotype accumulated approximately five and three times more shikimic acid, respectively, than the resistant biotype at 48 and 96 h after glyphosate application (Figure 2). These results are similar to those found for other glyphosate-resistant grass biotypes. In rigid ryegrass from Australia, the susceptible biotype accumulated two times more shikimic acid than the resistant biotype 48 h after exposure to glyphosate; however, the level of shikimic acid in the resistant biotype increased 10-fold, indicating that the herbicide is not excluded from its target site in vivo (Baerson et al. 2002a). A glyphosate-resistant rigid ryegrass biotype from California accumulated 10-fold less shikimic acid than the susceptible biotype at 11 d after the application of glyphosate at 2.24 kg ha^{-1} (Simarmata et al. 2003). In goosegrass, a glyphosate-resistant biotype accumulated approximately twofold less shikimic acid than the susceptible biotype 48 h after glyphosate application (Tran et al. 1999). In horseweed, no significant differences in shikimic acid levels were detected among the glyphosate-resistant and -susceptible populations 2 and 4 d after treatment; however, shikimic acid concentration decreased about 40% from 2 to 4 d after treatment in the resistant plants, but increased about 35% in the susceptible plants (Mueller et al. 2003).

Leaf-Segment Shikimic Acid Bioassay

The above results showed that shikimic acid accumulates to much lower levels after glyphosate treatment in the resistant biotype compared to the susceptible biotype. Similar results were found in a study that used excised leaf segments. Shikimic acid accumulated to the same level in leaf segments from both biotypes at 1,000 µM glyphosate, but the I_{50} for accumulate was much lower for the susceptible biotype (I_{50} = 8.1 ± 1.75 µM) vs. the resistant biotype (I_{50} = 101.8 ± 19.2 µM) (Figure 3). Results from both whole-plant and leaf-segment shikimic acid assays were consistent and demonstrated that the susceptible Italian ryegrass biotype accumulates more shikimic acid than the resistant biotype when treated with glyphosate.

EPSP Synthase Gene Sequencing

Partial *epsps* genes (1.2 kb cDNA) from the susceptible and the resistant Italian ryegrass biotypes were cloned and sequenced. The nucleotide sequence of the Italian ryegrass *epsps* gene showed over 97%, 90%, and 88% homology with the *epsps* genes of rigid ryegrass, goosegrass, and rice, respectively. Although the full-length *epsps* gene sequence of Italian ryegrass was not obtained, the sequenced region (80% relative to the rice *epsps* gene) included the region in

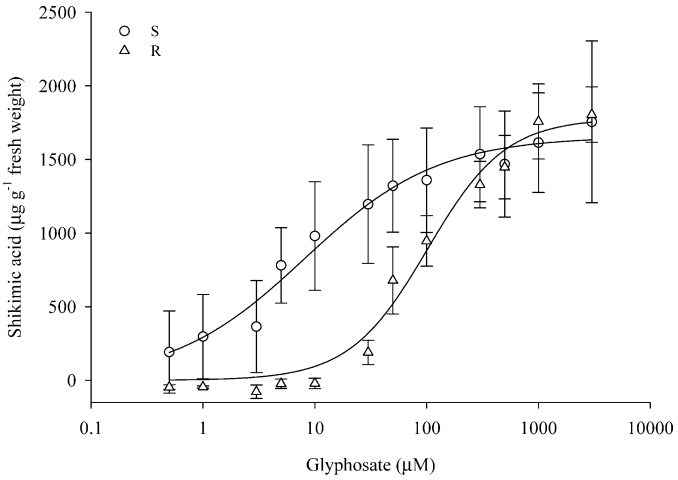

FIGURE 3. Shikimic acid accumulation in leaf segments of glyphosate-susceptible (S) and glyphosate-resistant (R) Italian ryegrass biotypes at different glyphosate concentrations. Vertical bars represent ± standard errors of the mean.

which point mutations conferring glyphosate resistance have been found. DNA sequence analysis of the *epsps* gene revealed no amino acid changes in the resistant Italian ryegrass biotype.

No accumulation of shikimic acid was observed in glyphosate-resistant soybean after herbicide treatment (Singh and Shaner 1998). In soybean, glyphosate resistance is conferred by the CP4 gene, which codes for an insensitive form of EPSP synthase (Padgette et al. 1996). Glyphosate resistance can also be conferred by single point mutations of the *epsps* gene, including glycine to alanine substitution at position 101 (Gly_{101} to Ala), or proline to serine substitution at position 106 (Pro_{106} to Ser) (Devine and Preston 2000). In goosegrass, the sequence comparison of the predicted EPSP synthase mature protein coding region from the susceptible and the resistant biotypes revealed two amino acid changes. One of these changes in the resistant EPSP synthase, Pro_{106} to Ser, contributed to reduced glyphosate sensitivity (Baerson et al. 2002b). In addition, a proline to threonine substitution at position 106 was also found to confer glyphosate resistance in goosegrass (Ng et al. 2003).

The mechanism responsible for herbicide resistance in Italian ryegrass is still unclear. However, the results on the accumulation of shikimate and the sequence of the *epsps* gene suggest that resistance is not due to an altered target site. Further studies, including glyphosate uptake, translocation, and metabolism of the herbicide will be conducted. Glyphosate resistance in rigid ryegrass is associated with an increased accumulation of the herbicide in the leaf tips and reduced accumulation in root tissues (Lorraine-Colwill et al. 2003) and meristematic zones (Wakelin et al. 2004). In horseweed, reduced glyphosate translocation plays a major role in resistance (Feng et al. 2004; Koger and Reddy 2005). However, it is well known that metabolism does not contribute to glyphosate resistance in rigid ryegrass (Feng et al. 1999; Lorraine-Colwill et al. 2003), goosegrass (Tran et al. 1999), or horseweed (Feng et al. 2004), suggesting that metabolic deactivation likely does not confer glyphosate resistance in Italian ryegrass.

Sources of Materials

[1] Sunshine Mix #1 potting mix, Sun Gro Horticulture, Inc., 110 110th Avenue NE, Suite 490, Bellevue, WA 98004.

[2] Glyfos®, isopropylamine salt of glyphosate, 356 g ae L^{-1}, Cheminova, Inc., Oak Hill Park, 1700 Route 23, Suite 300, Wayne, NJ 07470.

[3] RNeasy® Mini Kit, Qiagen Inc., 27220 Turnberry Lane, Suite 200, Valencia, CA 91355.

[4] Superscript™ III First Strand Synthesis System for RT-PCR, Invitrogen Corporation, 1600 Faraday Avenue, Carlsbad, CA 92008.

[5] Primus96 plus, MWG Biotech, Inc., 4191 Mendenhall Oaks Parkway, Suite 140, High Point, NC 27265.

[6] *Taq* DNA Polymerase (recombinant), Fermentas Inc., 7520 Connelley Drive, Unit A, Hanover, MD 21076.

[7] TOPO TA Cloning® Kit for Sequencing, Invitrogen Corporation, 1600 Faraday Avenue, Carlsbad, CA 92008.

[8] QIAquick® PCR Purification Kit, Qiagen Inc., 27220 Turnberry Lane, Suite 200, Valencia, CA 91355.

[9] ABI PRISM® 3771, Perkin-Elmer Applied Biosystems, 850 Lincoln Center Drive, Foster City, CA 94404.

[10] Sigma Plot®, Version 8.02, SPSS Inc., 233 South Wacker Drive, Chicago, IL 60606.

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